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DESIGN AND DEVELOPMENT OF PHYTOSOMES CONTAINING METHANOLIC EXTRACTS OF *NYMPHAEA NOUCHALI* AND *TRICHOSANTHES DIOICA*

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ABSTRACT

Phytosome represents a promising vesicular drug delivery system to deliver therapeutic compounds for a wide range of possible applications. *Nymphaea nouchali* (Nn), Indian Red Water Lily, is a water lily of genus *Nymphaea*. It is considered as a medicinal plant in Indian Ayurvedic medicine under the name *Ambal*; it was mainly used to treat indigestion and various ailments. *Trichosanthes dioica* (Td), cultivated widely in eastern India, has been used for overcoming problems like constipation, fever, skin infection, wounds, abdominal disorders and leprosy. Phytosome is obtained by reacting phosphatidyl ethanolamine (PE) in tetrahydrofuran with the selected botanical derivatives in dioxane:methanol (7:3) solvent system. Different Nn/Td:PE molar ratios (1:1, 1:2, 1:4, 1:6, 1:8 and 1:10) were employed using solvent evaporation technique. *In vitro* appraisal encompassed differential calorimetry, infra red spectroscopy, particle size, drug content, diffusion and stability studies. The results revealed that the optimized phytosomal carriers, PE(Nn/Td) exhibited the mean particle size of 268 nm and good *in vitro* stability in the ratio of 1:8. It also exhibited significant enhancement in diffusion rate compared to crude drug mixture and standard (Levimasole). Thus, the phytosomal carrier (PE) with 89 % of entrapped drug could be successfully tailored for Nn/Td with improved *in vitro* release characteristics which is promising for increasing drug delivery and decreasing the effect of exogenous factors.

KEYWORDS: *Nymphaea nouchali*, phosphatidyl ethanolamine, phytosomes, *Trichosanthes dioica*.

INTRODUCTION

The use of phytomedicines is widespread in most of the world's population. Over the past century, phytochemical and phytopharmacological studies have been performed on the plant extracts or/and products to establish their chemical composition and confirm the indications of traditional medicine. It has been observed that the separation and purification of the various components of an extract may lead to a partial loss of specific activity for the purified component. Most of the biologically active constituents of plants are polar or water soluble molecules. However, water soluble phytoconstituents (like flavonoids, tannins, terpenoids etc) are poorly absorbed either due to their large molecular size which cannot absorb by passive diffusion, or due to their poor lipid solubility; severely limiting their ability to pass across the lipid-rich biological membranes, resulting poor bioavailability. Phytosome is a patented technology developed by a leading manufacturer of drugs and nutraceuticals, to incorporate standardized plant extracts or water soluble phytoconsituents into phospholipids to produce lipid compatible molecular complexes and so vastly improve their absorption and bioavailability.[1-6]

Phytosome technology has been effectively used to enhance the bioavailability of many popular herbal extracts or active molecules including *Ginkgo biloba* extract, bilobalide isolated from *Ginkgo biloba*, silybin isolated from milk thistle (*Silybum marianum*), curcumin isolated from turmeric and green tea extract (*Camellia sinensis*) and can be developed for various therapeutic uses or dietary supplements. It is a complex between a natural product and phospholipids obtained by reacting stoichiometric amounts of each in an appropriate solvent. On the basis of their physical, chemical and spectroscopic characteristics, these complexes can be considered as novel entities.[7-11]

*Nymphaea nouchali*s, an aquatic plant is native to the Indian Subcontinent area. In its natural state, the red and blue water-lily is found in static or slow-flowing aquatic habitats of little to moderate depth. *Nymphaea nouchali var. cyanara* is the currently updated scientific name as per ICBN (International Code of Botanical Nomenclature). Recent experiments have confirmed that it has medicinal qualities as an antihepatotoxic and antidiabetic.[12-17] Like all water-lilies or lotuses, its tubers and rhizomes can be used as food items; they are eaten usually boiled or roasted. In the case of the red and blue water-lily, its tender leaves and flower peduncles are also valued as food.[18,19] Various secondary metabolites like sterols (nymphayol, isolated from flower), alkaloids, saponins, tannins, and flavonoids have been

isolated from this plant and these metabolites may be responsible for antibacterial activities. It has been reported to use in treatment of diabetes, tumor, inflammation, liver and urinary disorders, menstruation problems, indigestion and also used as food by the local people.

Trichosanthes dioica, (green potato) is a rich source of carbohydrates, vitamin A, vitamin C and other major nutrients and certain essential trace elements such as magnesium, potassium, copper, sulfur, and chlorine with that it seems to play an important role in human physiology. It also includes nicotinic acid, riboflavin, thiamine, 5-hydroxytryptamine, linoleic acid, oleic acid, colocynthin, saponin, trichosanthin, hntriacontane. The fruits are known to improve appetite and digestion; laxative; for disorders of circulatory system.[20-30] Thus the present study was undertaken to fabricate a novel drug delivery system for exploring the most effective immunomodulant activity of *Nymphaea nouchali* and *Trichosanthes dioica* in the treatment of disease related patients.

MATERIALS AND METHODS

Materials

The plant materials, *Nymphaea nouchali* and *Trichosanthes dioica* were procured from ABS Botanical Gardens, Salem, TamilNadu. Phosphatidyl ethanolamine was obtained as a gift sample from Sigma Aldrich, Bangalore. Dioxane, isopropyl alcohol, methanol, tetrahydrofuran and all other reagents and chemicals used were of analytical grade.

Preparation of plant extracts

The collected plant materials (Nn & Td) were cut into pieces and dried under shade at room temperature separately. The dried plant materials were coarsely pulverized to powder form and successively extracted with petroleum ether (60 – 70 ºC) for 8 h to remove fatty matters. The defatted marc was then subjected to soxhlet extraction with methanol to obtain the methanolic extract of the plant materials. It was then evaporated under reduced pressure to dryness to yield blackish brown colour extract of Nn/Td and stored in an air tight container.

Formulation of phytosomes

The phytosomes were prepared by combining the methanolic extracts of Nn and Td in an equimolar amount with various ratios of phospholipids using solvent evaporation technique. The ratio of drug and PE employed was ranges from 1:1 to 1:10. The accurately weighed quantity of PE was dissolved in tetrahydrofuran in a round bottomed flask, to which a specified quantity of the plant extracts dissolved in dioxane-methanol (7:3) mixture was

dispersed uniformly and refluxed for 3 h at 100 rpm. Then solvent was evaporated under reduced pressure at a temperature of about 60 ºC using a rotary flash evaporator resulting in the formation of thin film containing a solid mixture. Then phosphate buffer saline (PBS) pH 7.4 solution was added to the flask heated to about 50 ºC on a vortex, until a good dispersion of the mixture was obtained. The suspension was then sonicated for 10 min using 3 mm spindle in ultrasonicator.

Characterization of phytosomes

Differential scanning calorimetry (DSC)

Thermal bahaviour of the Nn/Td:PE complex was assessed using DSC analysis. The samples were sealed in an aluminium crimp cell and heated at a rate of 10 ºC/minute from 30 to 300 ºC in nitrogen atmosphere (60 mL/minute) to obtain the respective thermograms.

Fourier transform infrared spectroscopy (FTIR)

The drugs were analyzed by Fourier Transform Infra Red (FT-IR) Spectrophotometer. The FT-IR spectra for both the drug molecules (Nn and Td) were obtained by KBr pellet method. Spectral measurements were obtained by powder diffuse reflectance on a FT-IR spectrophotometer in the wave number region of 400–4000 cm^{-1}. Thus, the drugs were identified and characterized through the interpretation of their IR spectrums.

Surface Morphology

Approximately 5 µL of the phytosomal suspension was transformed to a cover slip, which in turn was mounted on a specimen tab. The samples were allowed to dry at room temperature. Then the particle size of the formulation was viewed and photographed using Scanning Electron Microscope (SEM). The particles were coated with platinum by using vaccum evaporator and thus, the coated samples were viewed and photographed in JEOL JSM-6701F Field Emission SEM.

Particle Size Distribution Analysis

The selected phytosomal suspension was subjected to laser particle counting method using Nanosizer for characterization of its size distribution. Here the sample was injected into the sample delivery port and allowed to pass through the controlling chamber. Then, suitable solvent was pumped through the chamber and a beam of laser light was allowed to fall on the sample cell. After required number of runs, they were directed towards the detector. From

this, the particle size range and the average mean particle size of the formulation was calculated.

Drug Content Analysis

For the determination of drug content, 5 mL of phytosomal suspension was swirled with 1 mL of 0.1 % triton X 100 for 1 hr and kept aside at room temperature for 24 h. Then, the resulted solution was analyzed spectrophotometrically at 278 nm after suitable dilution with phosphate buffer (PBS) pH 7.4.

Drug Entrapment Efficiency

The loading efficiency of phospholipids bonded phytosomes was determined by ultracentrifugation at 30,000 rpm for 20 min at 5 ^{O}C. The resulting supernatant solution was decanted and again centrifuged twice to remove the unentrapped drug molecules completely. The amount of drug entrapped in the phytosomes was determined by calculating the difference between the total amount of drug used to prepare the phytosomes and the amount of drug present in the aqueous medium.

$$\text{Encapsulation Efficiency} = \frac{\text{Actual drug content in Phytosomes}}{\text{Theoretical drug content}} \times 100$$

***In vitro* Diffusion Studies**

The *in vitro* release of drug from the phytosomal formulation was studied by using simple Franz diffusion cell apparatus which consists of an open ended cylindrical glass tube with an inner diameter of 2.5 cm, open at both ends. One end of the tube was tied with sigma dialysis membrane containing phytosomal suspension and placed in a beaker containing 400 mL of PBS pH 7.4, stirred at 200 rpm speed, maintaining the temperature at 37ºC. Periodically 5 mL of samples were withdrawn and after each withdrawal, same volume of medium was replaced. Then the samples were analyzed UV spectrophotometrically at 278 nm using PBS pH 7.4 as blank.

Stability Studies

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of variety of environmental conditions such as temperature, humidity and light. The preparations were divided into 3 sets and were stored at 4 ± 2 ^{0}C in refrigerator, at 25 ± 2 ^{0}C in room temperature and at 40 ± 2 ^{0}C / 70 ± 5 %

RH in Environment test chamber for 6 months and checked at regular intervals for changes in physical appearance. At each sampling period, the percentage of drug content was estimated spectophotometrically at 278 nm.

Statistical analysis

All the data were expressed as the mean ± standard deviation (S.D.) and statistical significance was analyzed using Student's *t*-test.

RESULTS AND DISCUSSION

Six different molar ratios (1:1, 1:2, 1:4, 1:6, 1:8 and 1:10) were employed in this study for screening the feasibility of complex formation. The work by Bombardelli and Patri referred the solvent evaporation technique as a possible method for drug:phospholipid complex formation.[31] In accordance with this theory, the solvent evaporation technique was applied in this study using dioxane-methanol mixture (7:3) as a volatile solvent system resulting in the formation of Nn/Td:PE complex. The composition of phytosomes containing methanolic extract of *Nymphaea nouchali* (Nn) and *Trichosanthes dioica* (Td) using phosphatidyl ethanolamine (PE) in different ratios was shown in **Table 1**.

Thermal analysis is one of the crucial tools to characterize the solid state of matter, particularly in complex form. DSC thermograms of Nn/Td (A), PE (B), Nn/Td:PE complex (1:8; C) and physical mixture (1:8; D) were obtained as depicted in **Figure 1**. The Nn/Td thermogram had shown a sharp endothermal peak at 283.5ºC, corresponding to Nn/Td melting. The PE thermogram demonstrated a sharp, pointed endothermal peak at 171.3ºC that could be attributed to the transition from gel state to liquid crystal state. In the physical mixture thermogram, the endothermal peaks of both Nn/Td and PE were clearly detectable.[32] The DSC thermogram of phytoformulation F5 had shown a broad endothermal peak at 167ºC which may be due to the formation of new complex.

Further investigation of complex formation was carried out using IR spectroscopy. **Figure 2** shows IR spectra of Nn/Td (A), PE (B), F5 phytosomes (C) and physical mixture (1:8; D). There was a slight difference between the physical mixture and the complex in the wavelength range from 1200 cm^{-1} to 900 cm^{-1} which corresponds to the region of phosphate group in PE. Also broadening of characteristic phenolic (-OH) group at 3500 cm^{-1} corresponds to the presence of hydrogen bonding. These observations suggested a weak

physical interaction between Nn/Td and PE during complex formation. Thus the IR spectra of all the phytosome formulations revealed that the ingredients were compatible with each other. Physicochemical characterization of PE(Nn/Td) phytosomes (F1, F2, F3, F4, F5 and F6) was depicted in **Table 1**. The formula F5 had shown lesser particle size (268 nm) compared to all other phytosomal formulations. The SEM photograph of the selected F5 formulation revealed the formation of smooth discrete vesicles as shown in **Figure 3**.

The diffusion of drug particles from its dosage form is a complex operation influenced by a number of factors. Differences in surface area, particle size and wetting properties may all play a role in affecting the diffusion rate of the drug. The *in vitro* diffusion profiles of the phytosome formulations F1, F2, F3, F4, F5 and F6; standard and crude drug were shown in **Figure 4** and **Figure 5**. From this study, the percentage of drug diffused into the medium was evaluated. The maximum percentage of drug diffused from the phytosomal formulations were obtained and found that F1, F2, F3, F4, F5 and F6 phytosomes attained the maximum release of 38.43 % after 6 h, 56.45 % after 12 h, 78.36 % after 13 h, 71.35 % after 12 h and 99.82 % after 17 h and 81.45 % after 13 h respectively. Thus, F5 phytosomes formulated with phosphatidyl ethanolamine in the ratio of 1:8 was found to attain maximum percentage of drug release with longer duration of time. Also, a significant enhancement in diffusion of the drug, Nn/Td was noted compared to crude extract and standard. This is because of the amphiphilic nature of the drug-phospholipid complex. Being an amphiphilic surfactant, PE could able to increase Nn/Td solubility by means of wetting and dispersion. Also the nano size of the phytovesicles might enhance the process of diffusion.

Table 1. Composition and characteristics of PE(Nn/Td) phytosomes

Formulations	Nn/Td:PE ratio	Drug content (% w/w)	Particle size (nm)	Entrapment efficiency (%)
F1	1:1	30.07 ± 0.02	567 ± 2.99	17.01 ± 0.07
F2	1:2	53.09 ± 0.13	330 ± 3.03	58.32 ± 0.11
F3	1:4	59.21 ± 0.03	476 ± 1.23	61.12 ± 0.22
F4	1:6	67.03 ± 0.12	389 ± 2.47	66.07 ± 0.13
F5	1:8	91.45 ± 0.23	286 ± 1.12	89.03 ± 0.26
F6	1:10	64.07 ± 0.19	347 ± 2.31	67.5 ± 0.13

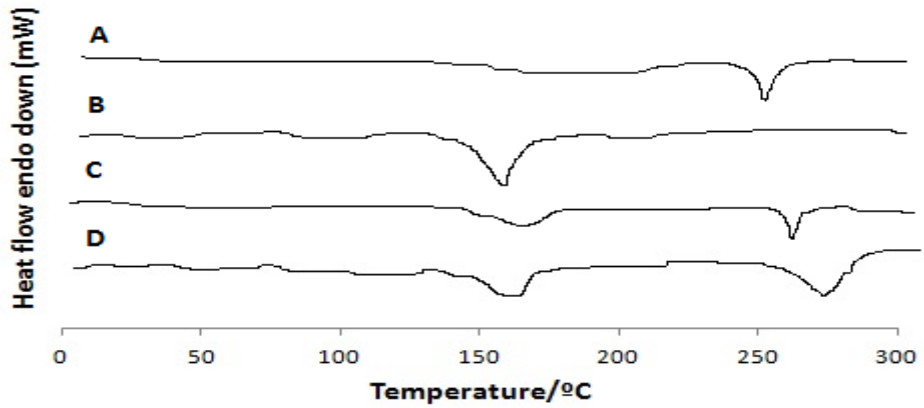

Figure 1. DSC thermograms of Nn/Td (A), PE (B), Nn/Td:PE complex (1:8; C), physical mixture (1:8; D)

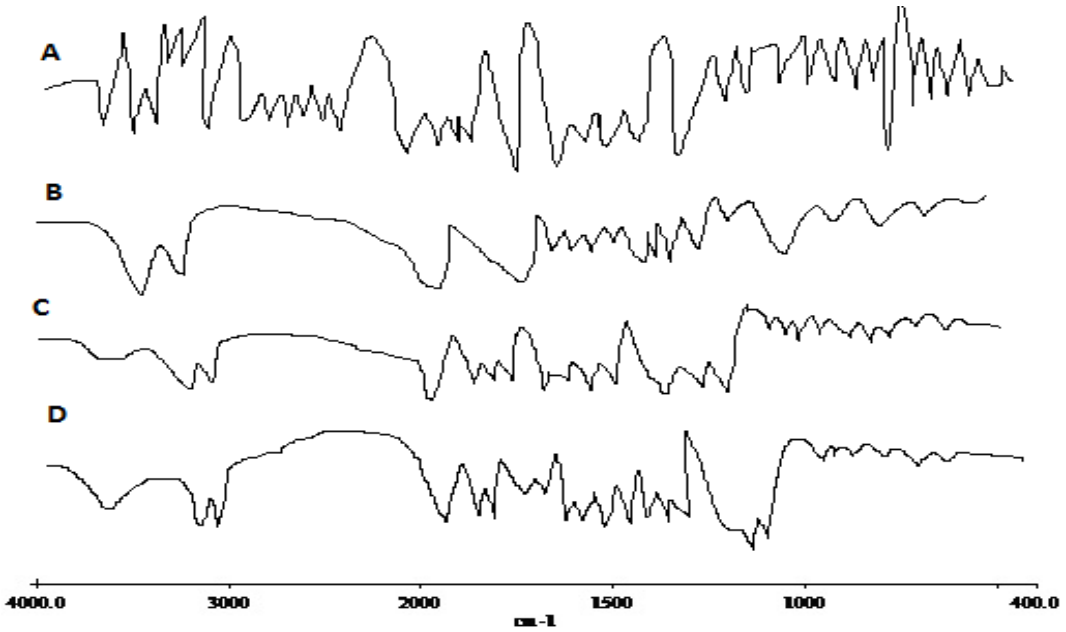

Figure 2. IR spectra of Nn/Td (A), PE (B), F5 phytosomes (C), physical mixture of Nn/Td:PE (1:8; D)

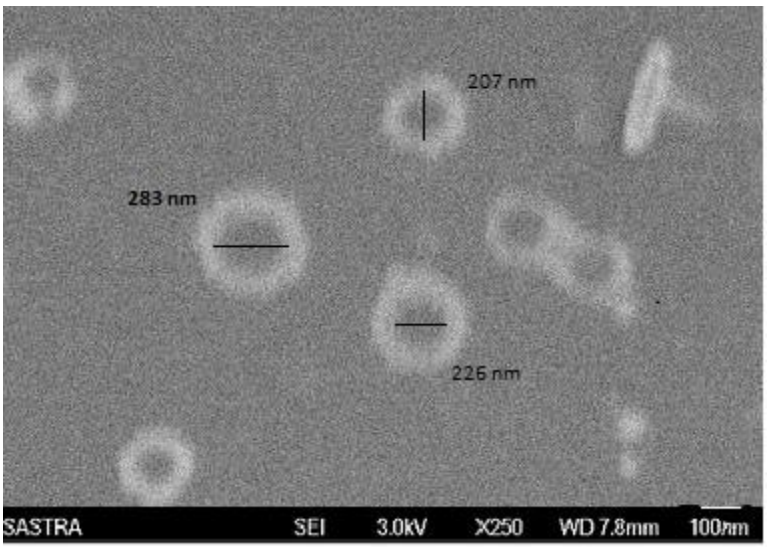

Figure 3. Scanning electron microscopic photographs of F5 phytosomes

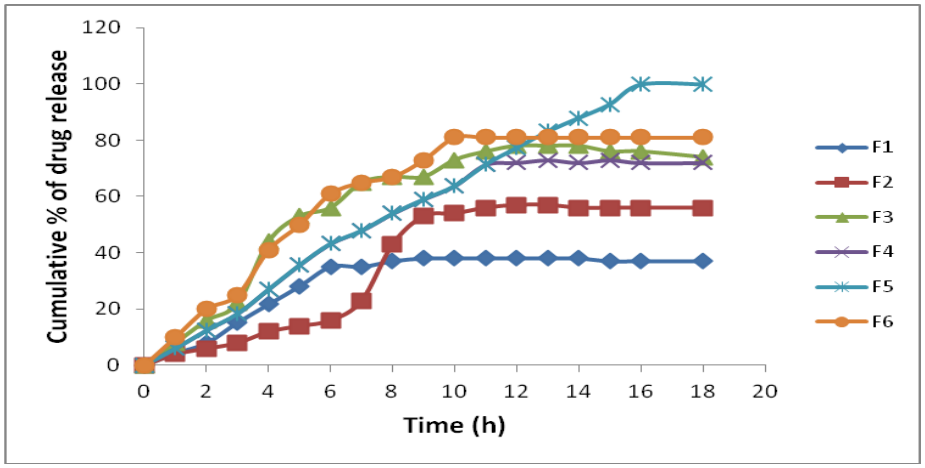

Figure 4. ***In vitro*** **diffusion release profile of phytosome formulations F1(1:1), F2(1:2), F3(1:4), F4(1:6), F5(1:8) and F6(1:10)**

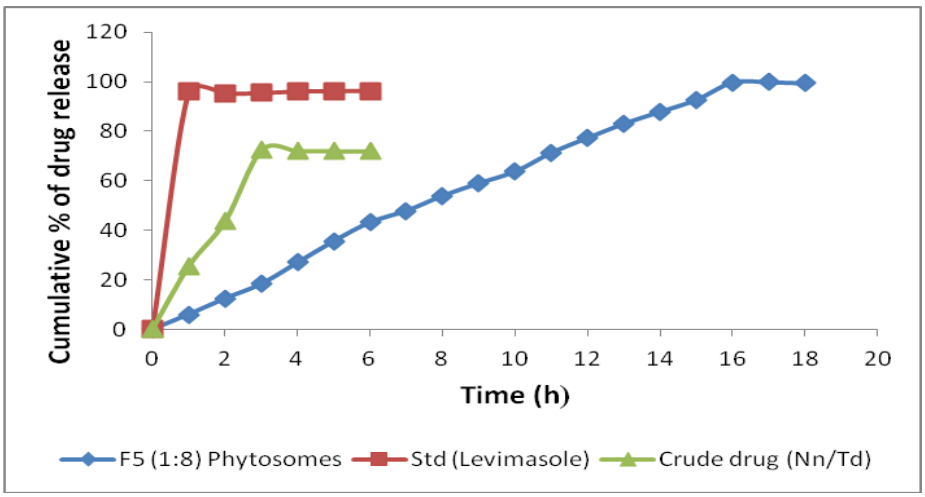

Figure 5. ***In vitro*** **release profile of crude drug (Nn/Td), standard drug (Levimasole) and F5 (1:8) phytosome formulation**

CONCLUSION

The present study was thus come out with a new phytovesicular carrier system for the delivery of Nn/Td phytoconstitutents. PE(Nn/Td) phytosomes could be successfully developed via the solvent evaporation technique. The amphiphilic phytovesicles were able to entrap 89.25 ± 0.34 % of drug, existing in spherical shape and nanometric range. The diffusion study results contended the ability of phytovesicles to enhance diffusion rate of the drug compared to its crude form and marketed standard. Thus, Nn/Td loaded phytovesicles tailored in this work exhibited promising physico-chemical properties paving the way for better drug delivery characteristics.

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